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TITLE: Potential Therapeutic Use of Relaxin in Healing Cranial Bone Defects

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14. ABSTRACT The overall objective is to provide proof-of-principle that recombinant human relaxin (rhRLX) administration will accelerate bone healing in a calvarial defect model in mice by promoting angiogenesis/vasculogenesis and osteogenesis, at least in part through incorporation of bone marrow-derived angio- and osteogenic progenitor cells into the lesion. Results from the initial study conducted during this reporting period demonstrated: successful production of chimeric mice after irradiation and GFP ⁺ bone marrow transplantation; reproducible implementation of uniform cranial lesions of ~1.5 mm diameter, and circulating concentrations of relaxin ranging from 30-80 ng/ml. However, after 11 weeks of healing, the lesion closure was comparable in the relaxin- and vehicle-treated mice (~70%). Therefore, in the next study we will decrease the healing time to 4 and 8 weeks in order to assess whether the closure occurs more rapidly with relaxin treatment. In view of the biphasic dose response curve for relaxin, which is dependent on the functional endpoint, we will also reduce the infusion rate by 20-fold to 0.05 microgram/h, which in our experience should produce a circulating relaxin concentration of 1-5 ng/ml.						
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1. Introduction

This DOD Discovery Award addresses the general problem of failed or delayed healing of craniomaxillofacial fractures. The objective is to provide proof-of-principle that recombinant human relaxin (rhRLX) administration will accelerate bone healing in a calvarial defect model in mice by promoting angiogenesis/vasculogenesis and osteogenesis, at least in part through incorporation of bone marrow-derived angio- and osteogenic progenitor cells into the lesion. This hormone/growth factor has numerous biological attributes that are likely to benefit bone fracture healing, and it has an excellent safety profile in humans. In brief, to test the hypothesis we use a cranial defect model in chimeric mice transplanted with GFP⁺ bone marrow. We follow defect closure by three dimensional microcomputed tomography (μ CT) and bone marrow progenitor cells at the lesion site by immunofluorescence. In addition, we quantitate bone growth and blood vessel density by histolomorphometry and immunohistochemistry, respectively.

2. Keywords

GFP⁺ chimeric mice, cranial defect closure, relaxin, angiogenesis, vasculogenesis, bone marrow-derived progenitor cells, 3-D microcomputed tomography, immunohistochemistry, immunofluorescence, flow cytometry

3. Accomplishments

A. Major Goals

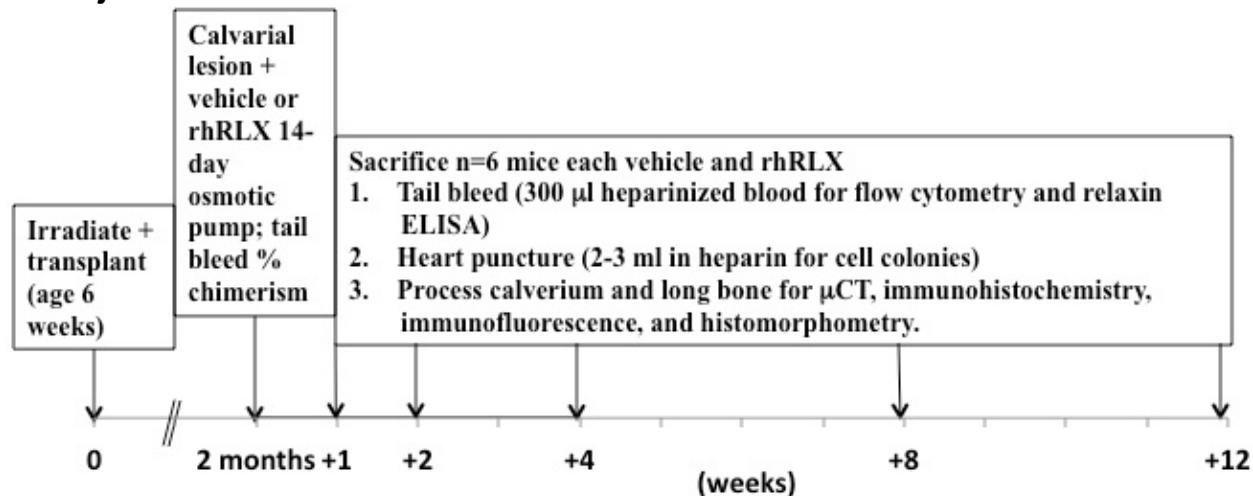


Figure 1. Potential therapeutic use of relaxin in healing cranial bone defects: Experimental Design

Major Tasks

1. Mouse manipulations
 - A. Subtasks
 - (i) Bone marrow transplantation
 - (ii) Calvarial lesion
 - (iii) Tail bleed
 - (iv) Osmotic pump implantation
 2. Necropsy

A. Subtasks

- (i) Tail blood
- (ii) Heart puncture and exsanguination
- (iii) Bone harvest
- (iv) Bone processing and fixation

3. Enumeration of circulating bone marrow angiogenic progenitor cells

A. Subtasks

- (i) Flow cytometry
- (ii) Colony forming units

4. Bone analyses

A. Subtasks

- (i) Three dimensional computed tomography
- (ii) Bone decalcification
- (iii) Bone histomorphometry
- (iv) Bone immunocytochemistry
- (v) Bone immunofluorescence

5. Data analysis and statistics

B. What was accomplished under these goals?

i. Major Activities

After irradiation and transplantation with purified bone marrow cells harvested from donor GFP⁺ mice followed by 2 months of recovery, bilateral cranial defects were created, and vehicle or relaxin was concurrently administered for 2 weeks. The 6 relaxin- and 5-vehicle-treated mice were euthanized 11 weeks after the cranial defect, and relaxin or vehicle treatment. For these 2 cohorts of mice, all Subtasks were completed under Major Task 1. Mouse Manipulation and Major Task 2. Necropsy. For Major Task 3, enumeration of circulating bone marrow angiogenic progenitor cells was accomplished by colony formation. For Major Task 4 (i) Three dimensional computed tomography was completed, while Subtasks (ii) Bone decalcification, (iii) Bone histomorphometry and (iv) Bone immunocytochemistry are in progress. (v) Bone immunofluorescence will be addressed contingent upon the results for subtask 4 (iv). We completed 5. Data analyses for cranial defect healing for this initial batch of mice, which we report herein.

ii. Specific Objectives

The specific objectives of this first series of experiments were: 1. to create GFP⁺ chimeric mice and by using flow cytometry to document the degree of chimerism achieved; 2. to reproducibly create bilateral, parietal defects of comparable diameter; 3. to chronically administer relaxin or vehicle by osmotic pump for 2 weeks and to measure circulating concentrations of relaxin by ELISA during the infusion; 4. to obtain blood from cardiac puncture followed by perfusion fixation under deep anesthesia and sacrifice of the mice 11 weeks after cranial defect; 5. to measure circulating bone marrow progenitor cells by flow cytometry and colonies; 6. to fix the bones for three dimensional computed tomography and measure cranial defect closure by three dimensional computed tomography; 7. to decalcify the bone for histomorphometry, immunohistochemistry and immunofluorescence.

iii. Significant Results

1. To create GFP^+ chimeric mice, and by using flow cytometry to document the degree of chimerism achieved.

Mice	% GFP signals	
	03/16/16	06/16/16
A1	54.5300%	40.5500%
A2	60.4200%	48.2400%
A3	70.7900%	39.2700%
A4	87.2300%	25.7900%
A5	77.1400%	88.3000%
A6	82.5000%	83.2200%
A7	54.2900%	90.1200%
A8	86.8700%	94.9200%
A9	43.8800%	89.8800%
A10	42.6500%	87.3800%
A11	39.2900%	88.2900%
Control mouse	0.0%--0.03%	

Table 1. % GFP+ Chimerism

As depicted in **Table 1**, we successfully produced chimeric mice. Approximately 2 months after the bone marrow transplantation, the percent GFP^+ chimerism of circulating leukocytes was $64 \pm 6\%$. At euthanasia approximately 3 months later, the percent GFP^+ chimerism was $71 \pm 8\%$. Not unexpectedly, some mice showed a decline in chimerism over time whereas others showed a rise. Nevertheless, the results are generally excellent and we will ultimately be able to normalize resident GFP^+ cells in the cranial defect to circulating values.

2. To reproducibly create bilateral, parietal defects of comparable diameter.

Using a dental burr and a variable speed drill, lesions of ~ 1.5 mm in diameter were reproducibly created (**Fig. 1**).

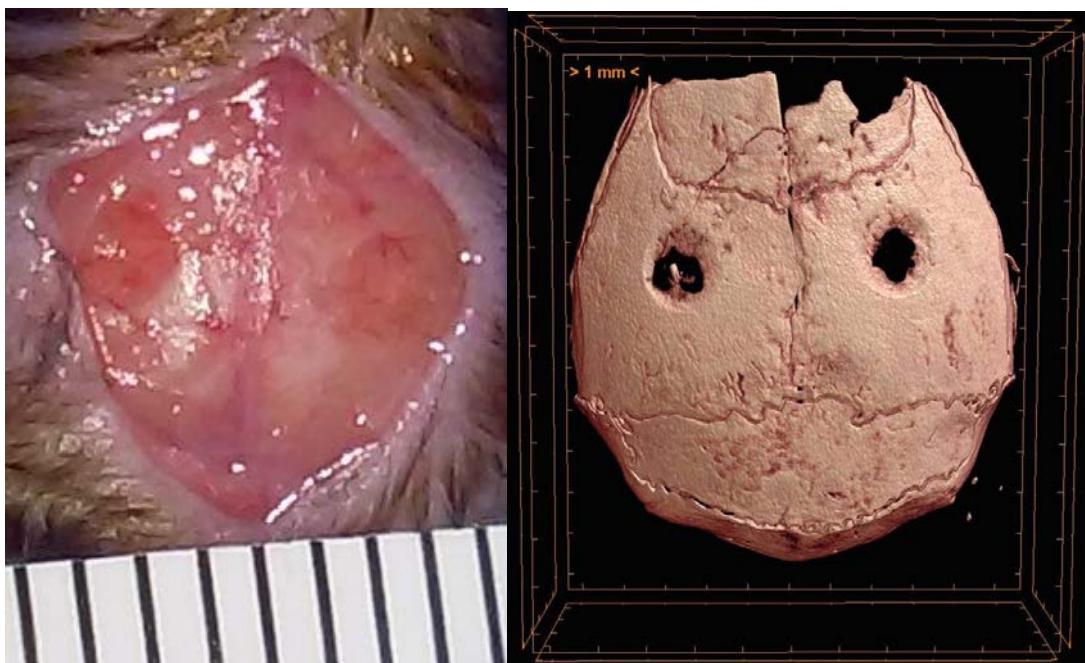


Figure 1. Representative photograph of bilateral, parietal lesions in a mouse during surgery. In the same mouse 11 weeks later, the cranial lesions were imaged by three dimensional computed tomography for measurement of defect closure.

3. To chronically administer relaxin or vehicle by osmotic pump for 2 weeks and to measure circulating concentrations of relaxin by ELISA during the infusion.

Values of 33, 37, 46, 89, 41 and 69 ng/ml of relaxin were measured in the 6 mice that were administered recombinant human relaxin. We did not test circulating levels in the vehicle-infused mice, because in our previous work, none was detected. This makes sense, because the ELISA we use does not detect mouse relaxin.

4. To obtain blood from cardiac puncture and perfusion fix under deep anesthesia and then euthanize the mice 11 weeks after cranial defect. Harvest crania and femur bones (control).

Completed.

5. To measure circulating bone marrow-derived angiogenic progenitor cells by flow cytometry and colonies.

In this initial experiment, we only measured circulating bone marrow-derived angiogenic progenitor cells by colony formation in cell culture. The reason is that we did not anticipate a difference between the relaxin- and vehicle-treated mice so remote from administration, which occurred starting with the cranial defect procedure 3 months earlier and lasted for 2 weeks thereafter. Nevertheless, we were interested to assess whether there might be a difference even at this late date. There was not: Relaxin: 2.75 ± 0.25 and vehicle 3.0 ± 0.71 (SEM) colonies/ml blood, $n=4$ pairs of mice. We measured colony formation rather than using flow cytometry, because the latter methodology requires considerably more time and effort.

6. To fix the bones for three dimensional computed tomography and to measure cranial defect closure by three dimensional computed tomography.

The lesions created on both the left and the right side demonstrated comparable degrees of closure (**Fig. 2**). In this initial trial, the mice administered relaxin and vehicle showed similar degrees of lesion closure, too (**Fig. 3**).

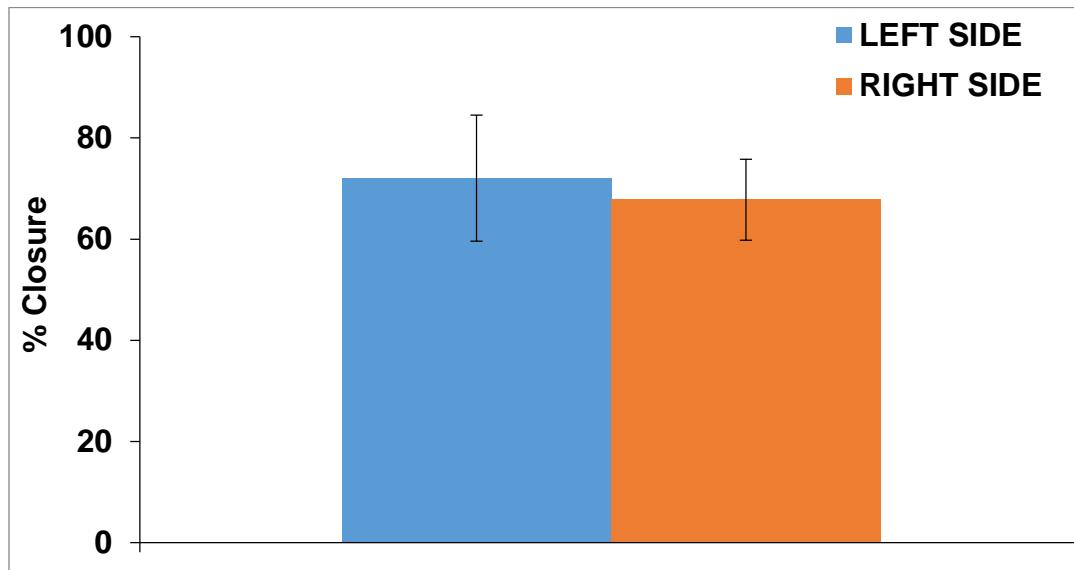


Figure 2. The percent closure for the left and the right cranial lesions was comparable after 11 weeks. $N=11$ mice. Mean \pm SD.

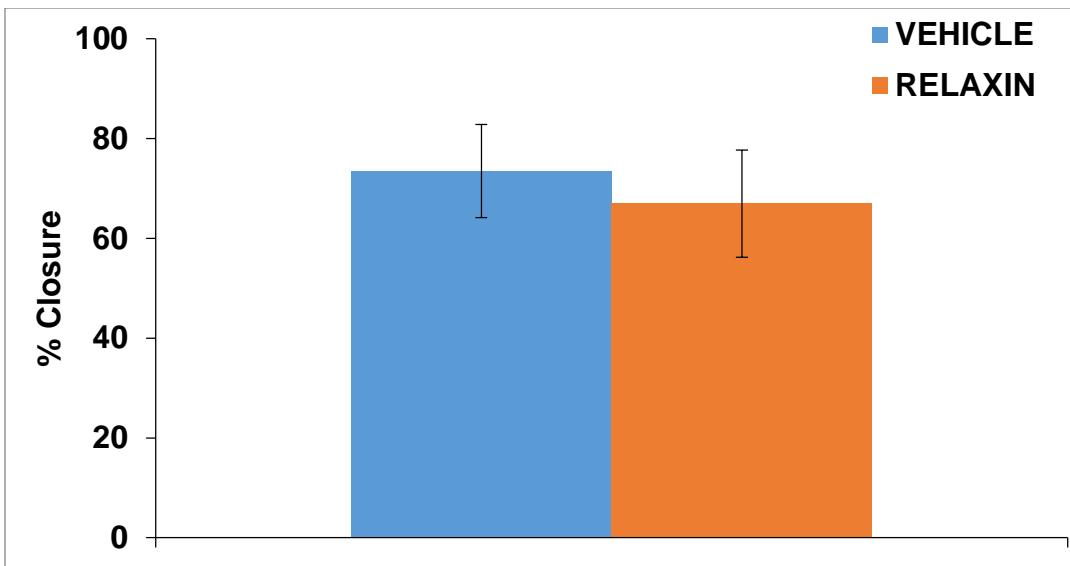


Figure 3. In this initial trial, the percent lesion closure was not significantly different between the relaxin (n=6) and vehicle (n=5)-treated mice 11 weeks after creating the lesions. Mean \pm SD.

7. *To decalcify the bone for histomorphometry, immunohistochemistry and immunofluorescence.*

The cranial bones are currently being decalcified.

iv. Other Achievements

Nothing to Report

C. What opportunities for training and professional development has the project provided?

Nothing to Report

D. How were the results disseminated to communities of interest?

Nothing to Report

E. What do you plan to do for the next reporting period to accomplish the goals?

1. The degree of closure was extensive in both the relaxin- and vehicle-treated mice (~70%), which may have masked a difference between the groups. That is, the relaxin-treated mice may have experienced a more rapid closure, which would also be therapeutically advantageous. Therefore, we will next investigate earlier time-points of 4 and 8 weeks.
2. We will forgo the irradiation and GFP⁺ BMT next time. These procedures add an additional 2-3 months to the protocol. Once we obtain a positive functional result, then we will reinstate irradiation and GFP⁺ BMT, in order to track bone marrow-derived progenitor cells.
3. The plasma concentrations of relaxin were higher than anticipated. Relaxin may have a biphasic dose response depending on the functional endpoint. Therefore, we will use a 20-fold lower dose of 0.05 microgram per hour, which should generate ~1-5 ng/ml in our experience.
4. Investigate lesion closure volume in addition to area.

4. Impact

A. What was the impact on the development of the principal discipline(s) of the project?

Nothing to Report

B. What was the impact on other disciplines?

Nothing to Report

C. What was the impact on technology transfer?

Nothing to Report

D. What was the impact on society beyond science and technology?

Nothing to Report

5. Changes/Problems

A. Changes in approach and reasons for change.

Nothing to Report

B. Actual or anticipated problems or delays and action or plans to resolve them.

The project start date was delayed for 2 reasons. First, I did not receive the relaxin (Serelaxin) for use in this study from Novartis until 09/24/15. This delay was caused by the inordinate amount of time that Novartis required to execute the MTA. I submitted the MTA 03/15/15. Second, I was not successful in hiring technical help until December 01, 2015. It took 2 rounds of postings and 2 interview sessions to find a qualified candidate. We will need to apply for a NCE at the end of the official award period.

C. Changes that had a significant impact on expenditures.

Nothing to Report

D. Significant changes in use or care of human subjects, vertebrate animals, biohazards, and/or select agents.

Nothing to Report

6. Products

Nothing to Report

7. Participants & Other Collaborating Organizations

A. What individuals have worked on the project?

(PI, Co-Investigators and Staff who committed at least 1 person month to the project)

Kirk P. Conrad, MD

Role: PI

Research ID: Kirk_Conrad

Nearest person month work: 1.0

Contribution to project: coordinated all research efforts of contributing Co-Is and staff; executed the surgical procedures of cranial lesions and osmotic pump implantations.

Yanpeng Diao, PhD

Role: Co-I

Research ID: NA

Nearest person month work: 1.2

Contribution to project: harvesting of bones from GFP⁺ mice for isolation of bone marrow cells, harvesting of bone marrow cells from bone, purification of bone marrow cells, heart puncture for blood collection and harvesting of long bones and crania, flow cytometry for % chimeric leukocytes, cell culture for colony formation.

Biswadeep Dhar

Role: Technician

Research ID: NA

Nearest person month work: 7.0

Contribution to project: assisted the PI in coordinating the research efforts of contributing Co-Is and Staff; animal husbandry; transport of mice to the various laboratories for procedures; coordination with Veterinary Staff for irradiation; organizing the tail injections and tail bleeds with the Animal Care Services Veterinary Technicians.

N.B. Mark S. Segal, MD/PhD Co-I, Joshua F. Yarrow, PhD Co-I and technician (VA Medical Center, Gainesville, FL), Ignacio Aguirre, PhD Co-I and technician also contributed, but less than 1 calendar month each.

B. Has there been a change in the active other support of the PD/PI(s) or senior key personnel since the last reporting period?

Nothing to Report

C. What other organizations were involved as partners?

Nothing to Report

8. Special Reporting Requirements

Nothing to Report

9. Appendices

Nothing to Report